STUDIES CONCERNING THE ACTION OF BISULFITE SOLUTIONS ON THE WHITE MOLD (*PENICILLIUM* AND *ASPERGILLUS* SP.P.) ISOLATED FROM AILING GRASS SILAGE

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INTRODUCTION

The production of good silage is automatic when all the requirements are satisfied at silo filling time. It often happens that the factors affecting the silage formation are not completely under control. Most trouble arises from the custom filling of silos. The equipment comes too late when the grasses are past their prime, or the operator rushes through the work leaving an ailing silage which heats and molds, faster than it can be fed. This is characteristic of too loosely packed silages because the air is not kept out.

Changes also occur in a well ensiled crop. The causes (Barnett, 1954) can be stated as: a. The continued respiration of the plant cells in the silage, giving off CO_2 and water, accompanied by heat evolution; b. production of acetic acid by Coliform bacteria which are destroyed by the heat developed in the early state of fermentation (These two stages have short duration and are completed in three days.); c. the lactic acid formation by the activity of lacto bacilli and streptococci on the available carbohydrates. These bacteria are more heat resistant. d. The fourth stage is the continuous formation of lactic acid which persists for 17 to 21 days. e. If not enough lactic acid is formed, *Clostridium butyricum* with *Clostridium welchii* attacks the lactic acid system, forming butyric acid (Barnett, 1954). These organisms are present in grass and soil and can be controlled by a pH of 4.2 or below (McCoy *et al.*, 1930). Butyric acid in different concentrations (Barnett and Miller, 1951) is relished by sheep. When in greater concentration this may be associated with a considerable degree of proteolysis with the formation of amides and ammonia which are not eaten well by ruminants. Similarly, molds (*Penicillia* sp.p. and *Aspergillae* sp.p.) which are characteristic of bad silage bring about proteolysis and ammonia production (Barnett, 1954) with heat evolution.

In 1952, Cowan, Bratzler and Swift announced the use of sodium metabisulfite instead of sulfur dioxide for the preservation of chopped grasses and corn.

The authors of the present paper carried out the study of the action of the bisulfite solutions on pure culture of white mold isolated from old dry silage and report in the experimental section which follows.

EXPERIMENTAL PROCEDURE AND RESULTS

The stock solutions of the following concentrations were prepared and were added to 15 ml. volume of the warm Sabouraud's agar medium according to tables

1. 1.25 percent sodium metabisulfite, alone.
2. 0.7 percent sodium metabisulfite and 0.7 percent lactic acid. Equal volumes were mixed.
3. 5.0 percent lactic acid, alone.
4. 2.5 percent sodium metabisulfite and 2.5 percent sodium lactate. Equal volumes were mixed, bringing the concentration to 1.25 percent of each.

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These concentrations were experimentally determined in advance so that they would fall in the range of their reactivity. The salt solutions were well mixed with the agar media to avoid layering before inoculation which may be disastrous on the spores transferred. The mixing was accomplished by quickly turning the test tubes, with the cotton plugs in, upside down several times. The agar media were kept at 55° C in a water bath while the stock solutions were at room temperature, 70° C, before mixing. Each tube was inoculated with .2 ml. of spores suspended in broth.

TABLE 1

The effect of 1.25% sodium metabisulfite concentrations and pH on the growth of molds of Penicillium *and* Aspergillus sp.p.

Volume in ml. of $Na_2S_2O_5$ added to 15 ml. Agar Media	Conc. of $Na_2S_2O_5$ in grams per L in Nutrient Agar Media	Osmotic Pressure in Atmospheres	Effect of Growth of Molds. No. of Colonies per 65 sq. cm.	pH
4	2.63	1.24	0	5.1
3	2.08	0.98	0	5.1
2	1.47	0.69	0	5.1
1.5	1.14	0.53	0	5.15
1.0	0.78	0.36	35	5.15
.75	0.59	0.27	65	5.2
.50	0.40	0.18	450	5.2
0.	0	0	500	5.2

TABLE 2

The combined effect of 0.7% sodium metabisulfite and 0.7% lactic acid concentration and pH on the growth of molds of Penicillium *and* Aspergillus sp. p.

Volume in ml. of $Na_2S_2O_5$ Added to 15 ml. Agar Media	Volume in ml. of Lactic acid Added to 15 ml. Agar Media	Ratio of Conc. of $Na_2S_2O_5$ and Lactic Acid in g/liter in Nutrient Agar Media	Total Osomotic Pressure in Atmospheres	Effect on Growth of Molds. No. of Colonies per 65 sq cm.	pH
2 ml.	2 ml.	0.74/0.74	0.52	0	4.3
1.5	1.5	0.60/0.60	0.42	0	4.45
1.0	1.0	0.41/0.41	0.29	4	4.6
.75	.75	0.32/0.32	0.23	120	4.7
.5	.5	0.22/0.22	0.15	500	4.9
.375	.375	0.16/0.16	0.11	500	5.0
.25	.25	0.11/0.11	0.08	500	5.1
0.	0.			500	5.2

The molds were isolated from spoiled silage by standard methods and grown on standard Sabouraud's agar media. The mold colonies appeared between 20 and 48 hours. Beyond this time no increase of colony-number occurred but an increase of colony-size took place. The number of colonies is taken as a measure of the effectiveness of the additive agents.

In bacteriological work, any mold contamination covers up bacterial growth by its quick development. Because molds were studied in this experiment, the slower development of any bacteria would be unlikely to affect the observation. Until the mold colonies grew together, the agar media stayed clear, free of bacteria growth. We considered the technique not perfect but adequate.

This study follows three lines of investigation: (1) toxic effect of these additive agents, (2) plasmolytic effect, and (3) pH.

Table 1 shows the effect of sodium metabisulfite alone. The lowest inhibiting concentration is 1.14 g/1. Decreasing concentration of metabisulfite results in increasing number of mold colonies.

Table 2 gives the toxic effect of sodium metabisulfite and lactic acid on the growth of molds. The presence of lactic acid reduces the inhibiting concentration of the metabisulfite from 1.14 g/1 alone in table 1, to 0.60 g/1 in the mixture.

TABLE 3

The effect of 5% lactic acid concentrations and pH on the Growth of molds of Penicillium *and* Aspergillus sp.p

Volume in ml. of Lactic Acid Added to 15 ml. Agar Media	Conc. of Lactic Acid in g/L in Nutrient Agar Media	Osmotic Pressure in Atmospheres	Effect on Growth of Molds. No. of Colonies per 65 sq. cm.	pH
4	10.5	2.60	0	2.95
3	8.33	2.08	0	3.05
2	5.88	1.48	0.6	3.2
1.5	4.54	1.13	500	3.55
1.0	3.13	0.78	500	3.6
.75	2.38	0.59	500	3.75
.50	1.61	0.40	500	4.0
0	0	0	500	5.2

TABLE 4

The combined effect of 2.5% sodium metabisulfite and 2.5% sodium lactate concentrations and pH on growth of the molds of Penicillium *and* Aspergillus sp. p.

Volume in ml. $Na_2S_2O_5$ Added to 15 ml. Agar Medium	Sodium Lactate	Ratio of Conc. of $Na_2S_2O_5$ and Sodium Lactate in g/L in Nutrient Agar Media	Osmotic Pressure in Atmospheres	Effect of Growth of Molds. No. of Colonies per 65 sq. cm.	pH
2 ml.	2 ml.	2.63/2.63	2.23	0	5.45
1.5	1.5	2.08/2.08	1.79	0	5.4
1.0	1.0	1.47/1.47	1.26	0	5.4
.75	.75	1.14/1.14	0.98	0	5.35
.50	.50	0.78/0.78	0.67	0	5.3
.375	.375	0.60/0.60	0.51	5	5.3
.25	.25	0.40/0.40	0.34	80	5.3
0	0	0	0	500	5.2

Table 3 gives the toxic effect of lactic acid alone. Here a concentration of 8.33 g/1 is required to inhibit mold growth completely.

Table 4 gives the toxic effect of sodium metabisulfite and sodium lactate. Here only 0.78 g/1 sodium metabisulfite are required to reach the inhibiting level. This is lower than that of sodium metabisulfite alone, (1.14 g/1) in table 1, but slightly higher concentration than that found in table 2 in which lactic acid is also present. Obviously, therefore, the combined effect of sodium metabisulfite and lactic acid is superior to the others.

The superior toxic effect of the combined solutions of sodium metabisulfite and lactic acid could be interpreted by the following chemical reactions.

(1) The change of one mole of sodium metabisulfite to two moles of sodium bisulfite. $Na_2S_2O_5 + H_2O \rightarrow 2\ NaHSO_3$

(2) The reaction of two moles of sodium bisulfite with two moles of lactic acid forming two moles of sodium lactate and one mole of sulfurous acid. The solubility of all the components in water points to the possibility of a reversible reaction as given below:

$$2\ NaHSO_3 + 2\ HO-\overset{\overset{\displaystyle O}{\|}}{C}-CHOH-CH_3 \rightleftarrows 2NaO-\overset{\overset{\displaystyle O}{\|}}{C}-CHOH-CH_3 + H_2SO_3$$

This indicates the presence of sulfurous acid in the chemical equilibrium.

Mixing equivalent sodium bisulfite and lactic acid produces a solution in which the bisulfite and the lactic acid are in ionic equilibrium with sodium lactate and sulfurous acid. This equilibrium is disturbed by any changes of any of the components of the solutions. Thus, H_2SO_3 decomposes into H_2O and SO_2 which may later disappear. Any other change of the sulfur compound may affect the reserve supply of biologically effective sulfur compounds.

In addition to the toxic effect of the stock solutions, a plasmolytic effect of these solutions was also considered. Osmotic pressure of 2.08 in atmospheres was taken as a threshold as shown in table 3, lactic acid alone, to be the minimum osmotic pressure to completely inhibit the growth of the molds. It is noted that all the other thresholds in tables 1, 0.53; 2, 0.42; and 4, 0.67 are far below this value. This may exclude the possibility that the inhibiting effect was caused by plasmolysis. These calculations are based only on the stock solutions added, not including the osmotic pressure of standard agar media.

The pH values were determined electrometrically with a glass electrode.

Alderman *et al.* (1955) in their discussion about bisulfite silages, attributed the preservative effect not to the H^+ion concentration as it was emphasized in the preparation of acid silages, but rather to the toxic effect of HSO_3^- ion. Rahn and Conn (1944), who studied the toxicity of ions received in the first and second steps of ionization of sulfurous acid, reported higher toxicity for the HSO_3^- ion and lower for the $SO_3^{=}$ ion. They reported the maximum concentration of HSO_3^- ions was obtained between pH 3.0 and 4.0. Even at pH 5.0, the concentration of HSO_3^- ions was 67 percent of the sulfurous acid present. These data of Rahn and Conn are in accord with pH measurement as reported in tables 1 and 2. In table 1, the pH values changed from 5.2 to 5.1 with increasing concentrations of the metabisulfite. In table 2, the pH values changed from 5.2 to 4.3 with increasing concentration of the mixture of sodium metabisulfite and lactic acid. In table 3, the pH values for different concentrations of lactic acid are given. In table 4, there was observed a slight increase in pH with increasing sodium metabisulfite and sodium lactate concentrations, for which a possible explanation could be the alkaline hydrolysis of sodium lactate. The increase in toxicity in table 4 over sodium metabisulfite alone, as shown in table 1, would indicate a potentiation of the sodium bisulfite by the presence of lactate.

Comparing the inhibiting concentrations of sodium bisulfite in grams per liter, that is, the lowest concentrations which inhibit mold growth completely, are given in:

Table 1	1.14 g/1 sodium bisulfite alone.
Table 4	0.78 g/1 sodium bisulfite and sodium lactate together.
Table 2	0.60 g/1 sodium bisulfite and lactic acid together.

The components of table 2 indicate the most effective agents for inhibiting the molds in question.

SUMMARY

Two types of molds were used in the test, *Penicillium* sp.p. and *Aspergillus* sp.p. The different ratios of sodium metabisulfite, lactic acid and sodium lactate have been experimentally determined. Sodium lactate has been found to increase the toxic effect of sodium metabisulfite but not as much as lactic acid. The combined toxic effect of sodium metabisulfite and lactic acid was superior to the others.

Concerning the hydrogen ion concentration, it was observed there was very little change in tables 1 and 4; in table 2 there was a ten fold increase; whereas in table 3 there was a hundred fold increase in the hydrogen ion concentration in column five.

When the acidity of the medium is approximately the same as that of the agar solution, the bisulifite alone is less effective than a mixture of bisulfite and sodium lactate.

Without bisulfite, the acidity by lactic acid must be about a hundred times that of the agar to inhibit mold growth.

Lactic acid and bisulfite in approximately equal molecular mixture effectively inhibit the mold growth at slightly more than half the concentration of bisulfite required without acid, with an increase in hydrogen ion concentration less than six fold.

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REFERENCES

Alderman, G., R. L. Cowan, J. W. Bratzler, and **R. W. Swift.** 1955. Some chemical characteristics of grass and legume silage made with sodium metabisulfite. Jour. Dairy Sci. **38**: **805**.

Barnett, A. J. G. 1954. Silage Fermentation. Academic Press Inc. p. 5, 57, 101.

——— and **T. B. Miller.** 1951. The laboratory determination of the protein digestibility of silage. Jour. Agri. Sci. 41: 317.

Cowan, R. L., R. W. Bratzler, and **R. W. Swift.** 1952. Use of sodium metabisulfite as a preservative of grass silage. Science 116: 154.

McCoy, E., E. B. Fred, W. H. Peterson, and **E. G. Hastings.** 1930. Cultural study of certain anaerobic butyric-acid-forming bacteria. Jour. Infectious Diseases 46: 118.

Rahn, O. and **J. E. Conn.** 1944. Effect of increase of acidity on antiseptic efficiency. Industrial Engineering and Chemistry 36: 185.